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Growth factors sustain primordial germ cell survival, proliferation and entering into meiosis in the absence of somatic cells

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Abstract

It is known that mammalian primordial germ cells (PGCs), the precursors of oocytes and prospermatogonia, depend for survival and proliferation on specific growth factors and other undetermined compounds. Adhesion to neighboring somatic cells is also believed to be crucial for preventing PGC apoptosis occurring when they loss appropriate cell to cell contacts. This explains the current impossibility to maintain isolated mouse PGCs in culture for periods longer than a few hours in the absence of suitable cell feeder layers producing soluble factors and expressing surface molecules necessary for preventing PGC apoptosis and stimulating their proliferation.

In the present paper, we identified a cocktail of soluble growth factors, namely KL, LIF, BMP-4, SDF-1, bFGF and compounds (*N*-acetyl-L-cysteine, forskolin, retinoic acid) able to sustain the survival and self-renewal of mouse PGCs in the absence of somatic cell support. We show that under culture conditions allowing PGC adhesion to an acellular substrate, such growth factors and compounds were able to prevent the occurrence of significant levels of apoptosis in PGCs for 2 days, stimulate their proliferation and, when LIF was omitted from the cocktail, allow most of them to enter into and progress through meiotic prophase I. These results consent for the first time to establish culture conditions for purified mammalian PGCs in the absence of somatic cell support and should make easier the molecular dissection of the processes governing the development of such cells crucial for early gametogenesis.

Keywords: Primordial germ cells; Growth factors; Apoptosis; *N*-acetyl-L-cysteine; Meiosis

Introduction

Early experiments using mouse primordial germ cells (PGCs) cultured without or onto cell feeder layers and in medium conditioned by different embryonic tissues, suggested that the neighboring cells produce a variety of compounds necessary for PGC survival and proliferation (De Felici and McLaren, 1982, 1983; Donovan et al., 1986; Godin et al., 1990). The possibility to isolate mouse PGCs and culture them under various culture conditions (De Felici, 1998), allowed to test the effects of several compounds on their survival and proliferation. This has led to the identification of growth factor candidates for these roles in vivo, including kit ligand (KL, also know as stem cell factor, SCF) (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991), leukemia inhibitory factor (LIF) (De Felici and Dolci, 1991; Matsui et al., 1991), basic fibroblast growth factor (bFGF or FGF-2) (Matsui et al., 1992; Resnick et al., 1992), bone morphogenetic protein-4 (BMP-4) (Pesce et al., 2002), stroma derived factor-1 (SDF-1, Molyneaux et al., 2003) and others (for a review, see De Felici et al., 2004). In addition, two potent non-growth factor mitogens for PGCs have been identified, namely forskolin (De Felici et al., 1993) and retinoic acid (Koshimizu et al., 1995).

Until now, however, all available culture methods for PGCs rely upon the presence of cell feeder layers (for a review, De Felici, 1998). Besides soluble growth factors and compounds, adhesion to cell monolayers in vitro and neighboring cells in vivo is believed to be crucial for optimal PGC growth (reviewed by De Felici et al., 2004). In

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fact, isolated PGCs recovered from 11.5 and 12.5 days post-coitum (dpc) gonads did not survive at 37°C without somatic cell support (De Felici and McLaren, 1983), and undergo rapid apoptotic degeneration (Pesce et al., 1993). The addition of single growth factors to the culture medium only slowed down PGC apoptosis and did not substitute for the cell feeder layers (Pesce et al., 1993; Pesce and De Felici, 1994). Together KL, bFGF and LIF have a remarkable effect on PGC cocultured onto certain somatic cell monolayers. Instead of ending proliferation, some PGCs continue to proliferate and form colonies of embryonic stem (ES) cell-like cells called embryonic germ (EG) cells (Matsui et al., 1992; Resnick et al., 1992; Moe-Behrens et al., 2003). The effect of combinations of soluble growth factors on purified PGCs cultured onto cell feeder layers has not been tested yet.

An adequate system for the culture of purified PGCs in the absence of cell feeder layers might have several obvious advantages. For example, it should reveal or clarify the extent to which processes crucial for PGC development such as proliferation, entering into meiosis, X chromosome reactivation and genomic imprinting erasure are autonomous properties of PGCs, rather than being dependent on interactions with somatic cells. In addition, proliferating cultures of PGCs without the undefined and variable contribution of cell feeder layers should allow to determine direct effects of compounds on such cells and would greatly facilitate, for example the molecular dissection of the mechanisms controlling PGC mitotic cycle and the shift from mitosis to meiosis, so far little understood in mammals and of central importance for gametogenesis.

Materials and methods

PGC isolation and culture

PGCs were obtained from sex undifferentiated gonadal ridges of 11.5 dpc CD-1 mice embryos (Charles River, Italy) following the EDTA-puncturing method (De Felici and McLaren, 1983) and collected directly in the culture medium (see below). The purity of PGCs after such isolation, determined by alkaline phosphatase (APase) staining (see below), was approximately 80%. In some experiments, PGCs were purified using the MiniMACS immunomagnetic cell sorter (PGCs purity > 90%) (Pesce and De Felici, 1995). Embryos were sexed by RT-PCR genotyping for the YMT2/B locus (Bouma et al., 2004).

About 500 (EDTA-puncturing) and 5000–8000 (MiniMACS sorting) PGCs were seeded onto a Transwell Falcon cell culture polyethylene terephtalate (PET) membrane filter (Falcon, cat. n. 353095) inserted into a 24 well plate Falcon tissue culture dish (cat. n. 353504) in a total of 0.9 ml (0.2 ml and 0.7 ml above and below the membrane, respectively) of high glucose D-MEM (Gibco), containing non-essential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM glutamine, 0.25 mM pyruvate, 75 mg/L penicillin-G, 50 mg/L streptomycin purchased from Sigma and 15% fetal calf serum (Gibco). Recombinant murine KL (SCF), human bFGF, murine SDF-1 and human BMP-4 were from R&D System; ESGRO murine LIF was from Calbiochem and *N*-acetyl-L-cysteine, forskolin and retinoic acid from Sigma. Growth factors and compounds were added at the time of seeding and when indicated changed every day of culture. Cultures were carried out in a humidified incubator at 37°C and 5% CO_2 in air. In some experiments, the cultures were carried out in the modular incubator chamber (MIC.101) (ICN Biomedicals) pre-gassed with an atmosphere of 5% O_2 and 5% CO_2 and placed in a humidified incubator.

STO cells (an embryonic mouse fibroblast cell line purchased from ATCC, USA) and Sl^4m220 cells (embryonic fibroblasts from $Sl^{-/-}$ mice stably expressing membrane-bound mouse KL, Flanagan et al., 1991; a kind gift from P. Donovan, Johns Hopkins University School of Medicine, Baltimore) were propagated in D-MEM plus 10% FCS (see, above). Confluent cell monolayers were inhibited from proliferation by treatment with mitomycin C (Sigma, 10 μg/ml, 3 h, 37°C). Cells were then harvest by trypsinization and frozen at a concentration of about 5×10^6 cells/ml. After thawing, cells were plated at concentration of $2–2.5 \times 10^5$ cells/ml in 24-well Falcon tissue culture dish.

Analysis of PGC viability, apoptosis and proliferation

Before seeding and after 1–3 days of culture, PGCs were identified and counted by labeling for APase or TG-1 antibody as reported in De Felici (1998).

The viability of PGCs in culture was determined in parallel replicates using the Erythrosin B exclusion test as reported by De Felici and McLaren (1983). Briefly, after removal of the culture medium, a solution of the dye in PBS (0.4 mg/ml) was added and after 5 min at room temperature, the number of viable (i.e., non-stained) cells was scored in a minimum of 8 fields (30 cells per field) under a 20× objective of an inverted microscope. Cells in the withdrawal medium or grown in suspension (see below) were also tested for viability after centrifugation using an hemocytometer chamber (De Felici and McLaren, 1983). The viability of PGCs cocultured onto cell feeder layers was evaluated by morphological criteria after APase staining, since it was unfeasible to distinguish viable PGCs attached to the cell monolayers using the dye exclusion test.

The number of apoptotic PGCs was scored by dual staining with APase and TUNEL cytochemistry. Cells were washed in M_2 medium, fixed in 70% ice-cold ethanol (5 min) and stained for APase as described above. The culture were washed with PBS and stained for TUNEL-positive cells according to protocol of the POD (Peroxidase Detection) in situ cell death kit (Roche Molecular Biochemical, cat. n. 1684817). APase and APase/TUNEL-positive cells were scored in random fields as reported above using a light transmitted microscope. PGCs in

suspension were collected in the culture medium and attached to a poly-L-lysine-coated slide (De Felici, 1998) before the APase/TUNEL assay.

The BrdU incorporation assay was performed on attached PGCs (see Results and discussion) after 24 h of culture. Briefly, the culture medium was replaced with fresh medium containing 1:1000 dilution of BrdU labeling reagent (Amersham) and cells incubated for an additional 2 h, fixed in ice-cold 70% ethanol (5 min) and stained for APase as described above. The culture were washed with deionized water and stained for BrdU according to the manufacture instructions. APase and APase/BrdU positive cells were scored in random fields as reported above.

Evaluation of PGCs entering into and progression through meiotic prophase I

The capability of PGCs to enter into meiosis was evaluated using dual immunostaining with TG-1 (a monoclonal antibody similar to anti SSEA-1, known to bind to the surface of undifferentiated PGCs, Wylie et al., 1986) and anti-SCP-3 antibody (a polyclonal antibody that recognizes the meiosis specific synaptonemal complex protein termed Cor1/Sycp3, Di Carlo et al., 2000; Chuma and Nakatsuji, 2001). Cells in culture were fixed and treated as described in Chuma and Nakatsuji (2001). Briefly, after fixation, cells were incubated over night at 4°C in 1:150 dilution of TG-1 (a kind gift from P. Donovan, Johns Hopkins University School of Medicine, Baltimore) and 1:200 anti-SCP-3 (a kind gift from Christer Höög, Karolinska Institute, Stockolm). TRITC-conjugated anti-mouse IgM and FITC-conjugated anti-rabbit IgG were used as secondary antibodies.

The meiotic prophase stages were determined by characteristics patterns of SCP-3 immunolabeling in squashed and spread cells as described in Mahadevaiah et al. (2001).

Western blotting analysis

For Western blotting analysis, cells were lysed in a solution containing 50 mM Tris, 150 mM NaCl, 20 mM EDTA, 1% deoxicholate, 0.1% SDS, 1 mM PMSF, 20 μ/ml leupeptin and 1 mM Na vanadate. Proteins (about 30 μg) were subjected to 10% SDS/PAGE electrophoresis. After transfer to Hybond C nitrocellulose membranes, blots were treated according to standard procedures. Probing of the blots was performed using goat anti mouse LIF (Santa Cruz, cat. n. sc1336) (1:100) for 3 h at room temperature. After a second 1 h incubation period with anti-goat peroxidase-conjugated antibody, blots were developed for the peroxidase reaction.

Statistical analysis

All experiments were replicates at least three times. The means were tested for homogeneity of variance, and analyzed by ANOVA. The level of significance was set at $P < 0.05$ and $P < 0.01$.

Results and discussion

Combined action of growth factors, forskolin, retinoic acid and N-acetyl-L-cysteine sustains PGC survival by preventing apoptosis and stimulates PGC proliferation

Previous studies have shown that several growth factors alone or in combination positively influence the survival and/or proliferation of PGCs cultured onto suitable cell feeder layers (for a review, see De Felici et al., 2004). In the present paper, in a first series of experiments, we tested the effect of single growth factors, namely KL, LIF, SDF-1, bFGF and BMP-4 on the survival of purified 11.5 dpc PGCs. To compare the growth factor effect with that of soluble compounds produced by STO embryonic fibroblasts, generally employed as PGC cell feeder layers (De Felici, 1998), PGCs were cultured onto a Transwell membrane (see Materials and methods) placed into a well containing culture medium or in which the insert separated them from STO cell monolayers grown at confluence 24 h before on the bottom of the well (termed herein STO-CM). In this last case, half medium was replaced before PGC seeding. In parallel experiments, PGCs were seeded directly onto STO cell monolayers, a standard culture condition for mouse PGCs (termed herein STO-D).

It is to be pointed out that the Transwell membranes were employed also because PGCs were observed to attach to such substrate. In fact, while PGCs do not ordinarily attach to tissue culture dishes (De Felici and McLaren, 1983), under our culture conditions after 5 h either in the absence of growth factors or in the presence of single growth factor, they showed 58% ± 2.3 adhesion to the membrane inserts, an adhesion level similar to that onto STO cell monolayers (De Felici and Pesce, 1994). Despite such adhesion, however, the viability tests showed that after 24 and 48 h of culture, none of the tested growth factors was able alone to support PGC survival over the control values. When 100 ng/ml KL was added to the culture medium, a constant, albeit not statistically significant, increase of the number of viable PGCs was observed. On the contrary, as expected, PGCs cultured in STO-CM or under STO-D conditions showed similar adhesion levels (62 ± 5.0), but a significant higher survival (Fig. 1). We then tested the combination of all growth factors, each employed at the ED_{50} value reported to positively affect the growth of PGCs cocultured onto cell monolayers (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1992; De Felici and Dolci, 1991; Matsui et al., 1991; Resnick et al., 1992; Pesce et al., 1993; Molyneaux et al., 2003). The growth factor cocktail increased significantly PGC adhesion in comparison to control (80% ± 3.2 plus growth factors vs. 58% ± 2.3 control) and, as showed in Fig.

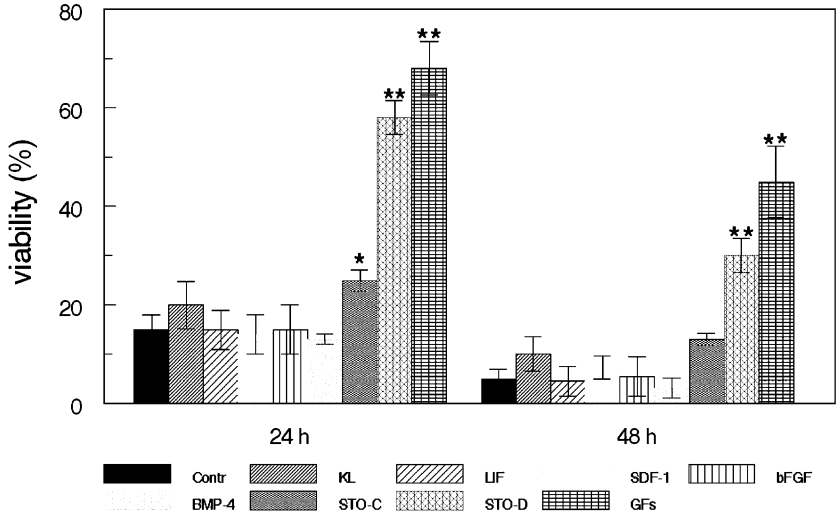

Fig. 1. The effect of growth factors and STO cell feeder layers on the viability of mouse PGCs cultured onto Transwell PET membrane filter. Contr = control; 100 ng/ml KL = kit ligand; 1000 UI/ml LIF = leukemia inhibitory factor; 20 ng/ml bFGF = basic fibroblast growth factor; 50 ng/ml BMP-4 = bone morphogenetic protein-4; 20 ng/ml SDF-1 = stroma derived factor-1; STO-CM = STO cell conditioned medium; STO-D = PGCs attached onto STO cell monolayers; GFs = all growth factors employed together at half of concentrations reported above. Each column represents the mean of at least three experiments ± SE. * = $P < 0.05\%$; ** = $P < 0.01\%$.

1, exerted a prosurvival action higher than STO-CM and STO-D conditions.

Since it has been recently reported that antioxidants considerably increase the survival of pig PGCs cultured in the absence of cell feeder layers (Lee et al., 2000), in the aim to determine if also mouse PGC survival was improved by antioxidants and if the growth factor pro-survival action increased, we tested the effect of the *N*-acetyl-L-cysteine (NAC) on the survival of isolated PGCs cultured with or without the growth factor cocktail. While NAC did not affect PGC adhesion (not shown), at a concentration of 1 mg/ml, it significantly increased PGC survival after 24 h. This effect decreased after 48 h and was absent at 72 h of culture (Fig. 2A). Moreover, the survival of PGCs cultured in medium containing the growth factor cocktail plus 1 mg/ml NAC was significantly higher than that of PGCs cultured in the presence of the growth factors alone (Fig. 2B). Even, in this condition, however, the number of surviving PGCs dropped after 3 days of culture.

In order to evaluate whether decreased oxygen tension was able to mimic the effect of NAC, PGC cultures were carried out in 5% instead that in standard 21% O_2. The reduced oxygen tension alone or in combination with the growth factors did not result in any significant increase of PGC survival after 24 h of culture (data not shown).

As expected, the pro-survival effect of the growth factors plus NAC was associated with a marked reduction of PGC apoptosis both at 24 and 48 h of culture as estimated by the TUNEL assay (Fig. 3).

Similar levels of PGC survival and apoptosis were detected in cultures in which PGCs were purified by the MiniMACS method (data not shown).

In order to determine whether the pro-survival/anti-apoptotic effect of the growth factor cocktail plus NAC was dependent on PGC adhesion to the membrane insert (see above), PGCs were seeded onto membranes pre-coated with a thin film of 2% agar in PBS pre-adsorbed with the culture medium for about 12 h. Under this condition, PGCs attachment to the filter was completely prevented and the most part of them formed small aggregates. After 24 h of

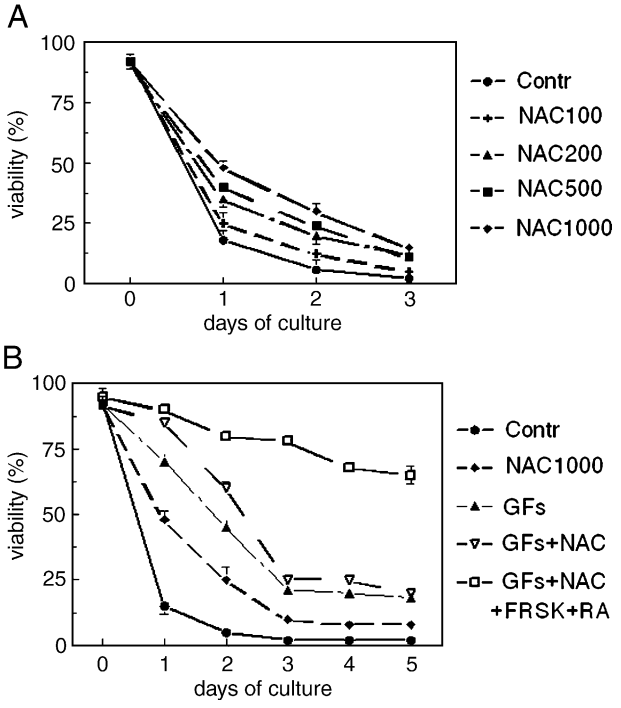

Fig. 2. Effect of different concentration of *N*-acetyl-L-cysteine (NAC) (μg/ml) on PGC viability during 3 days of culture alone (A) or of 1000 μg/ml NAC in combination with GFs reported in Fig. 1 or of 1000 μg/ml NAC with GFs and 5 μM forskolin and 1 μM retinoic acid in culture medium changed every day.

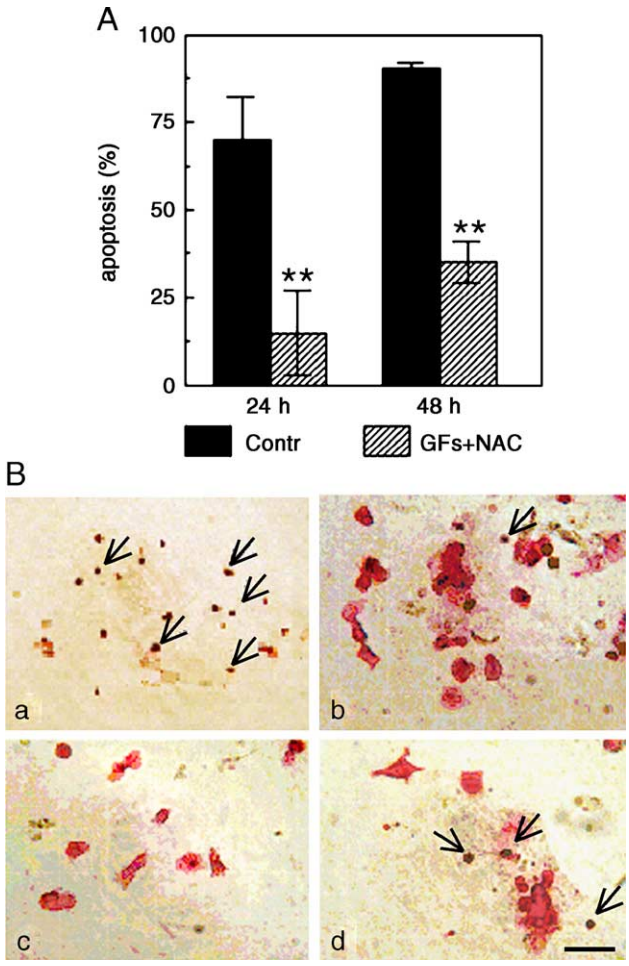

Fig. 3. (A) Combination of growth factors (GFs) and NAC reported in Fig. 2, reduces significantly the percentage of PGC apoptosis in culture as determined by TUNEL cytochemistry. (B) Representative microscopic fields of PGC in culture after dual staining for APase and TUNEL; a = control PGCs cultured in plain medium, b–d = PGCs cultured in the presence of medium supplemented with GFs and NAC, note in part (a) APase positive PGCs with reduced diameter and nuclear TUNEL staining (arrows) indicative of apoptosis while in panel (b–d) only a few PGCs show such features (arrows). ** = $P < 0.01\%$. Scale bar = approximately 35 μm.

culture, these cells despite the presence of growth factors plus NAC showed a low level of viability (<30%) and a high percentage of apoptosis (>80%). Thus, indicating that growth factors are ineffective on PGCs in suspension and that adhesion to an acellular substrate is crucial although not sufficient for maintenance of PGC survival. In fact, as reported above, relatively high level of adhesion to the filter (around 60%) was alone unable to sustain PGC survival for 24 h. Experiments are carried out to characterize the factors responsible of PGC adhesion to the filter and the role of growth factors and serum in such process.

On the basis of the results obtained in previous studies, it is likely that some of the growth factors present in the cocktail tested in the present paper rather or in addition to exert anti-apoptotic effect stimulate PGC proliferation. For example, bFGF and BMP-4 have been reported to stimulate PGC proliferation, while KL, LIF and SDF-1 are likely to be mainly anti-apoptotic factors (for a review and references, see De Felici et al., 2004). Although in the present paper we did not specifically address this question, we were interested to evaluate the proliferation of PGCs under the different culture conditions employed.

By scoring the number of PGCs after 24 h in medium containing growth factors plus NAC (386/well ± 43), we found that it did not change significantly in comparison to that at the beginning of culture (432/well ± 55). The addition of 5 μM forskolin and 1 μM retinoic acid, two potent mitogens for PGCs (De Felici et al., 1993; Koshimizu et al., 1995), increased the PGC number up to 10–15% the control values.

Finally, using the BrdU incorporation assay, we evaluated that after 24 h about 50% ± 5.3 PGCs cultured in the presence of the growth factor cocktail were in S phase, this percentage increased up to 70% ± 2.5 in the presence of forskolin and retinoic acid and was markedly lower, around 5% ± 1.5 and 27% ± 3.2, in STO-CM and STO-D conditions, respectively. Thus, confirming the besides inhibiting apoptosis, the growth factor combination employed by us is likely to exert mitogenic effect on PGCs and that forskolin and retinoic acid are potent mitogens for PGCs rather than anti-apoptotic compounds. It is likely that the reason for the absence of increase of PGC number in the presence of growth factors plus NAC is that under such condition PGC proliferation only compensates for the loss of cells due to apoptosis and the lack of complete attachment to the membrane. Moreover, for the reason described in the next section, it is also possible that a fraction of PGCs in S phase are cells fated to enter meiosis rather than complete the cell cycle with mitosis.

By daily changing the GF-containing medium plus NAC, forskolin and retinoic acid, we were able to significantly increase the number of surviving PGCs up to 5 days of culture (Fig. 2B). In such condition, however, we observed as from 3 days of culture a rapid growth of the few contaminating somatic cells present at the beginning of culture.

LIF prevents PGCs to enter into meiosis and favors their self-renewal

After entering into the gonadal ridges, PGCs undergo 2–3 further rounds of mitosis, and by 12.5 dpc in both female and male embryos, they enter a pre-meiotic stage and upregulate meiotic genes such as *Scp3* (Di Carlo et al., 2000; Chuma and Nakatsuji, 2001). In the fetal testis, meiosis proceeds no further and PGCs enter mitotic arrest as GO/G1 prospermatogonia. In the fetal ovary, in contrast, PGCs enter meiotic prophase as oocytes, and pass through leptotene, zygotene and pachytene stages before arresting in diplotene at about the time of birth.

It has been reported that mouse PGCs cultured onto Sl^4-m220 cell monolayers, producing constitutively high

amount of the membrane bound KL, enter into meiosis irrespective of the sex as estimated by the expression of the meiosis specific protein of the synaptonemal complex SCP-3 (Chuma and Nakatsuji, 2001). Moreover, Chuma and Nakatsuji showed that PGC entering into meiosis was markedly inhibited by LIF. In an attempt to reproduce these results, we cultured 11.5 dpc PGCs under the same culture conditions used by these authors that do not include the addition of any soluble growth factors to the culture medium. After 2–3 days of culture, however, we observed very few PGCs (less than 10%) with the characteristic dot-like SCP-3 staining inside the nuclei, indicative of the beginning of meiosis. Western blotting analysis revealed that our Sl^4-m220 cell monolayers as well as at lesser extent STO cells, expressed high levels of LIF (Fig. 4A). In light of the reported inhibitory effect of LIF on the PGC entering into meiosis (Chuma and Nakatsuji, 2001), this offers a likely explanation of our failure to reproduce the

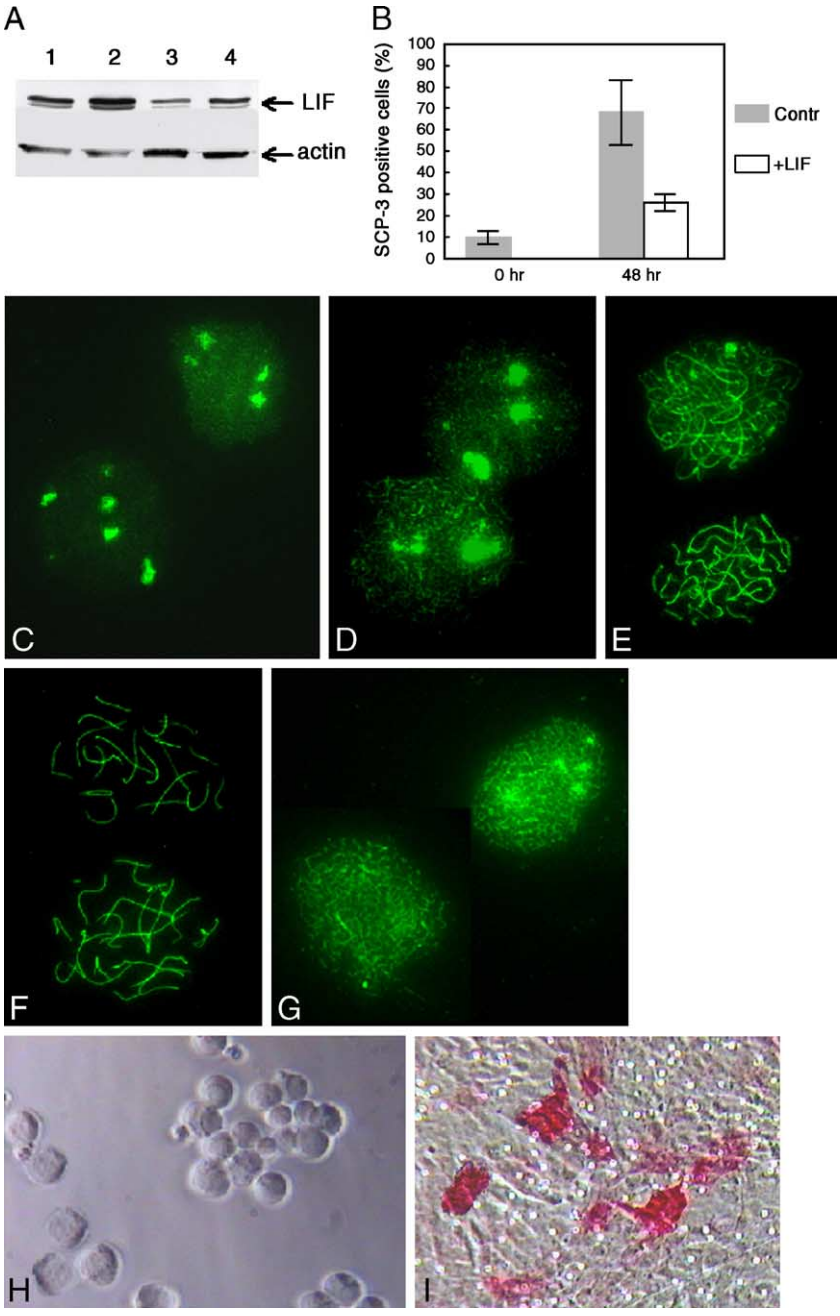

Fig. 4. (A) Production of LIF by Sl^4-m220 (lanes 1 and 2, mitomycin-treated cells) and STO (lanes 3 and 4, mitomycin-treated cells) cell monolayers detected by Western blotting analysis; (B) Capability of PGCs to enter into meiosis in culture and inhibitory effect of 1000 UI/ml LIF as estimated by SCP-3 staining, ** = $P < 0.01\%$; (C–F) Stages of meiotic prophase I in cultured female PGCs as identified by SC labeling patterns using anti-SCP-3 antibodies: (C) preleptotene/leptotene, (D) leptotene, (E) zygotene, (F) pachytene and (G) early diplotene. (H) Aggregates of meiotic female PGCs formed after 5 days of culture; (I) Aggregates of APase positive EG-like cells formed onto gonadal somatic cells after 7 days of culture of male 11.5 dpc PGCs.

Table 1
Stage of meiotic prophase I as determined by the characteristics patterns of SCP-3 immunolabeling in squashed and spread germ cells after 2–7 days (d) of culture of 11.5 dpc PGCs

Meiotic stage	Female PGCs			Male PGCs		
	2d	4–5d	7d	2d	4–5d	7d
P/L	84 ± 5.5	3 ± 1.5	–	95 ± 2.5	5 ± 2.8	–
L	16 ± 3.2	70 ± 2.9	–	5 ± 5.5	70 ± 3.9	–
Z	–	18 ± 2.7	–	–	25 ± 6.2	–
P	–	9 ± 1.7	45 ± 3.2	–	–	62 ± 2.8
D	–	–	55 ± 4.2	–	–	38 ± 3.2

Numbers represent the mean percentage ± standard error of three scores performed in two or three independent experiments on a total of at least 200 cells; SCP-3 positive cells showing clear morphological signs of degeneration were excluded from the score.

Chuma and Nakatsuji's results. We then analyzed the capability of purified 11.5 dpc PGCs cultured onto Falcon insert in the presence of the growth factor combination reported above with or without LIF plus NAC, forskolin and retinoic acid to enter meiosis. Fig. 4B shows that under such conditions after 48 h of culture about 68% of either female and male 11.5 dpc PGC exhibited SCP-3 staining with characteristic dot-like signals inside the nuclei typical of the preleptotene–leptotene stage of the prophase I (Di Carlo et al., 2000; Chuma and Nakatsuji, 2001). We observed that during the following days of culture (up to one week), most of the surviving PGCs tended to form aggregates which detached from the filter (Fig. 4H). The formation of cell aggregates was directly related to the number of cells seeded being evident over 5000 PGCs/dish. Spreading preparations of these aggregates from both male and female germ cells showed various prophase I stages, ranging from leptotene–zygotene to zygotene–pachytene stages and early diplotene (Figs. 4C–G, Table 1). While we did not observe significant difference between female and male PGCs in the capability to enter into meiosis and reach the pachitene stage, male germ cells showed lower capability to progress beyond pachitene (Table 1). Studies are in progress to further analyze differences between female and male PGCs and to determine if critical processes of prophase I such as DNA double-strand breaks and repair, synapsis and recombination of homologous chromosomes correctly occur in this culture condition. Moreover, our results open the way to the possibility to produce growing oocytes from PGCs using a multi-step in vitro culture system of diplotene oocytes onto granulosa cells (Klinger and De Felici, 2002).

When 1000 UI/ml LIF was added to the medium, the percentage of PGCs entering into meiosis was significantly decreased to less than 30% (Fig. 4B). Removal of other growth factors or forskolin or retinoic acid did not affect PGC entering into meiosis. We observed that SCP-3 staining was generally associated with reduced or absence of TG-1 staining and that in the presence of LIF the number of PGCs showing TG-1 staining was increased. This suggests that LIF favors PGC self-renewal. In cultures carried out up to 7–10 days mainly in the presence of LIF, we observed small aggregates of APase/TG-1 positive cells attached to the filter resembling small EG cell colonies (Fig. 4I). However, these colonies did not appear to grow further and their EG cell identity remains to be established.

In conclusion, in the present paper, we report for the first time that mouse PGCs in culture survive, proliferate and enter into meiosis in virtual absence of somatic cells. This was achieved by preventing apoptosis in purified PGCs by a combination of soluble growth factors and the antioxidant *N*-acetyl-L-cysteine for a time much longer than that reported in previous studies. It is likely that activation of the apoptotic program in PGCs depends on a variety of conditions and that its efficient prevention requires signaling by several growth factors. Under the culture conditions employed by us, the anti-apoptotic signals generated by growth factors require PGCs attachment to an acellular substrate and remain efficient for several days. The capability of PGCs to proliferate at high rate, to undergo meiosis and even to progress and complete meiotic prophase I under the in vitro condition reported here is remarkable and should make easier to identify the molecular pathways regulating these processes particularly the shift between mitosis and meiosis, which is so far difficult to study in such cells.

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References

Bouma, G.J., Hart, G.T., Washburn, L.L., Recknagel, A.K., Eicher, E.M., 2004. Using real time RT-PCR analysis to determine multiple gene expression patterns during XX and XY mouse fetal gonad development. Gene Expr. Patt. 5, 141–149.

Chuma, S., Nakatsuji, N., 2001. Autonomous transition into meiosis of mouse fetal germ cells in vitro and its inhibition by gp130-mediated signalling. Dev. Biol. 229, 468–479.

De Felici, M., 1998. Isolation and culture of germ cells from the mouse embryo. Cell Biology: A Laboratory Handbook, vol. 1. Academic Press, San Diego, pp. 73–85.

De Felici, M., Dolci, S., 1991. Leukemia inhibitory factor sustains the survival of mouse primordial germ cells cultured on TM_4 feeders layers. Dev. Biol. 147, 281–284.

De Felici, M., McLaren, A., 1982. Isolation of mouse primordial germ cells. Exp. Cell Res. 142, 476–482.

De Felici, M., McLaren, A., 1983. In vitro culture of mouse primordial germ cells. Exp. Cell Res. 144, 417–427.

De Felici, M., Pesce, M., 1994. Interactions between migratory primordial germ cells and cellular substrates in the mouse. In: Marsh, J., Goode, J. (Eds.), Germline Development, Ciba Foundation Symposium, vol. 182, pp. 140–159.

De Felici, M., Dolci, S., Pesce, M., 1993. Proliferation of mouse primordial germ cells in vitro: a key role for cAMP. Dev. Biol. 157, 277–280.

De Felici, M., Scaldaferri, M.L., Lobascio, M., Iona, S., Nazzicone, V., Klinger, F.G., Farini, D., 2004. In vitro approaches to the study of primordial germ cell lineage and proliferation. Hum. Reprod. Updat. 10, 197–206.

Di Carlo, A.D., Travia, G., De Felici, M., 2000. The meiotic specific synaptonemal complex protein SCP3 is expressed by female and male primordial germ cells of the mouse embryo. Int. J. Dev. Biol. 44, 241–244.

Dolci, S., Williams, D.E., Ernst, M.K., Resnick, J.L., Brannan, C.I., Lock, L.F., Lyman, S.D., Boswell, H.S., Donovan, P.J., 1991. Requirement for mast cell growth factor for primordial germ cell survival in culture. Nature 352, 809–811.

Donovan, P.J., Stott, D., Cairns, L.A., Heasman, J., Wylie, C.C., 1986. Migratory and postmigratory mouse primordial germ cells behave differently in culture. Cell 44, 831–838.

Flanagan, J.G., Chan, D.C., Leder, P., 1991. Transmembrane form of kit ligand growth factor is determined by alternative splicing and is missing in Sl^d mutant. Cell 64, 1025–1035.

Godin, I., Wylie, C.C., Heasman, J., 1990. Genital ridges exert long-range effects on mouse primordial germ cell numbers and direction of migration in culture. Development 108, 357–363.

Godin, I., Deed, R., Cooke, J., Zsebo, K., Dexter, M., Wylie, C., 1991. Effects of the *steel* gene product on mouse primordial germ cells in culture. Nature 352, 807–809.

Klinger, G.F., De Felici, M., 2002. In vitro development of growing oocytes from fetal mouse oocytes: stage-specific regulation by stem cell factor and granulosa cells. Dev. Biol. 244, 85–89.

Koshimizu, U., Watanabe, M., Nakatsuji, N., 1995. Retinoic acid is a potent growth activator of mouse primordial germ cells in vitro. Dev. Biol. 168, 683–685.

Lee, C.K., Weaks, R.L., Johnson, G.A., Bazer, F.W., Piedrahita, J.A., 2000. Effects of protease inhibitors and antioxidants on In vitro survival of porcine primordial germ cells. Biol. Reprod. 63, 887–897.

Mahadevaiah, S.K., Turner, J.M.A., Baudat, F., Rogakou, E.P., de Boer, P., Blanco-Rodriguez, J., Jasin, M., Keeny, S., Bonner, W.M., Burgoyne, P.S., 2001. Recombinational DNA double-strand breaks in mice precede synapsys. Nat. Genet. 27, 271–276.

Matsui, Y., Toksoz, D., Nishikawa, S., Williams, D., Zsebo, K., Hogan, B.L., 1991. Effect of steel factor and leukaemia inhibitory factor on murine primordial germ cells in culture. Nature 353, 750–752.

Matsui, Y., Zsebo, K., Hogan, B.L., 1992. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70, 841–847.

Moe-Behrens, G.H., Klinger, F.G., Eskild, W., Grotmol, T., Haugen, T.B., De Felici, M., 2003. Akt/PTEN signaling mediates estrogen-dependent proliferation of primordial germ cells in vitro. Mol. Endocrinol. 12, 2630–2638.

Molyneaux, K.A., Zinszner, H., Kunwar, P.S., Schaible, K., Stebler, J., Sunshine, M.J., O'Brien, W., Raz, E., Littman, D., Wylie, C., Lehmann, R., 2003. The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. Development 130, 4279–4286.

Pesce, M., De Felici, M., 1994. Apoptosis in mouse primordial germ cells: a study by transmission and scanning electron microscope. Anat. Embryol. 189, 435–440.

Pesce, M., De Felici, M., 1995. Purification of mouse primordial germ cells by MiniMACS magnetic separation system. Dev. Biol. 170, 722–725.

Pesce, M., Farrace, M.G., Piacentini, M., Dolci, S., De Felici, M., 1993. Stem cell factor and leukemia inhibitory factor promote primordial germ cell survival by suppressing programmed cell death (apoptosis). Development 118, 1089–1094.

Pesce, M., Klinger, F.G., De Felici, M., 2002. Derivation in culture of primordial germ cells from cells of the mouse epiblast: phenotypic induction and growth control by Bmp4 signalling. Mech. Dev. 112, 15–24.

Resnick, J.L., Bixler, L.S., Cheng, L., Donovan, P.J., 1992. Long-term proliferation of mouse primordial germ cells in culture. Nature 359, 550–551.

Wylie, C.C., Stott, D., Donovan, P.J., 1986. In: Browder, L. (Ed.), Developmental Biology: A Comprehensive Synthesis, vol. 2. Plenum Press, New York, pp. 433–439.